



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>4</sup> :</b> <b>A61K 39/12, C12N 7/00</b> <b>// A61K 35/76</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 89/ 06542</b> <b>(43) International Publication Date:</b> 27 July 1989 (27.07.89)
<b>(21) International Application Number:</b> PCT/GB89/00047 <b>(22) International Filing Date:</b> 18 January 1989 (18.01.89) <b>(31) Priority Application Number:</b> 8801338 <b>(32) Priority Date:</b> 21 January 1988 (21.01.88) <b>(33) Priority Country:</b> GB  <b>(71) Applicant (for all designated States except US):</b> QUADRANT BIORESOURCES LIMITED [GB/GB]; Manor Farm, Soulbury, Leighton Buzzard, Bedfordshire (GB). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> ROSER, Bruce, Joseph [AU/GB]; The Old Vicarage, Church Lane, Balamsham, Cambridgeshire (GB). <b>(74) Agent:</b> ABLEWHITE, Alan, James; Marks & Clerk, 57-60 Lincoln's Inn Fields, London WC2A 3LS (GB).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), BR, CH (European patent), DE (European patent), FR (European patent), GB, GB (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), SU, US.  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title: PRESERVATION OF VIRUSES</b>  <b>(57) Abstract</b> <p>A method of preserving live viruses comprises subjecting an aqueous system containing the virus to drying either in the frozen state or at ambient temperature, in the presence of trehalose.</p>		

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PRESERVATION OF VIRUSES

This invention relates to the preservation of live viruses and in particular to the drying of live viruses in a stable form from which they can be reconstituted while retaining their immunogenic or other useful activity..

Live viruses have a number of important uses. The most important is the use of viruses, either intact or attenuated, as immunogenic vaccines. Two good examples are polio virus and measles virus. However, live virus vaccines are extremely difficult to maintain under storage. They cannot, at the moment, be dried and reconstituted without losing their immunogenic effect. Similarly, they cannot be frozen, for the same reason. Consequently, virus vaccines are required to be kept in aqueous media under cool sterile conditions, for example in refrigerators. One of the diseases mentioned above, measles, is of great epidemiological importance, especially in the third world. Each year in Africa alone millions of children die of measles. This is largely because the vaccines necessary for prevention cannot be distributed in the majority of countries concerned which do not have the infrastructure or wealth

to provide point-of-use refrigeration.

There is thus a desperate need worldwide for a simple means of preserving viruses in an intact immunogenic form which can be kept without refrigeration or other elaborate control, and which can be reconstituted simply with water just before use.

Other viruses have considerable importance in other fields. For example, Epstein Barr virus (EBV) is of considerable importance in the preparation of human B-cell lines to produce monoclonal antibodies and for studies on human molecular genetics. EBV at present has to be stored in aqueous media under refrigeration.

Similarly, bacteriophages such as phage Lambda derived from E. coli, are of importance in manipulation of DNA and the production of gene libraries in the so-called genetic engineering. Again, this virus must be kept under aqueous conditions under refrigeration.

While it is well-known that drying of unstable biological products, either at room temperature or in the frozen state (lyophilisation) can be aided by adding stabilizing agents to the product in question, there has been no report to our knowledge of the successful stabilization of live viruses.

Thus, for example, UK patent application 2009198A describes the stabilization of meningococcal polysaccharides under lyophilisation, by combining them with various sugars including sucrose, raffinose, glucose and trehalose. In this case, of course, the antigenic component is not a live virus. Similarly, UK Patent application 2126588A describes the stabilization of Tumor Necrosis Factor in the presence of certain sugars and sugar acids.

In European Patent application 140489A1, rubella virus antigen is stabilized by immersion in certain sugars. Again, however, no possibility of stabilizing the virus itself is mentioned.

Our own earlier British patent application 2187191A (WO87/00196) describes and claims the preservation of various proteins and other macromolecules to drying at ambient temperature, by drying in the presence of trehalose. Dead virus vaccines are mentioned, but there is no indication that the immunogenic and other functions of live viruses can be preserved in this way.

We have now found that the presence of trehalose in the viral medium during drying either frozen or at ambient temperature, enables live viruses to be preserved and subsequently reconstituted while retaining

substantially all their immunogenic properties or other useful properties, including viability. Thus, for the first time, the possibility is provided of having stable dry formulations of vaccines such as polio and influenza viruses.

According to the present invention there is provided a method of preserving live viruses comprising subjecting an aqueous system containing the virus to drying either in the frozen state or at ambient temperature, in the presence of trehalose.

In general, the more trehalose added the better, although in practice beneficial effects are obtained if the aqueous system contains from 1 to 20% by weight of trehalose, typically 5 to 10% by weight. Naturally, the amount of trehalose added will, in part, depend on the amount of virus present in the system, but the exact ratio of trehalose to virus is not particularly critical.

While the virus system can then be lyophilized successfully using standard techniques, to provide a dry material which can be stored at ambient temperatures for subsequent reconstitution with water, it is in fact possible to dry the aqueous system at ambient temperatures without loss of immunogenic or other activity. Thus, for example, an aqueous preparation containing bacteriophage lambda GT10 and 10% by weight

trehalose was dried at room temperature (about 20°C) and was subsequently reconstituted by addition of an aqueous suspension of E. coli in an aqueous medium.

The following examples illustrate the invention further:-

Example 1

EVALUATION OF THE EFFECT OF TREHALOSE ON THE  
PRESERVATION OF EPSTEIN BARR VIRUS AT ROOM TEMPERATURE  
IN THE DRY STATE

Materials and Methods.

1. Virus production.

The B95.8 producer cell line (The Epstein Barr Virus, Ed. Epstein and Achong, Springer Verlag, Berlin 1979) was grown to confluence in DMEM/10% FCS. 30ng/ml of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was added, and the culture maintained at 33°C for 10 days. The supernatant was collected, clarified through a 0.45 µm filter and concentrated by ultrafiltration (300 Kdalton nominal cut off) of 40x relative to the original culture.

## 2. Drying and Storage

To aliquots of the concentrated virus equal volumes of preservative (2%, 20% or 40% trehalose (Sigma) in water) was added, and half of each solution stored at 4°C. The other half was freeze dried in a glass ampoule and stored at room temperature. After 1 week the freeze dried material was reconstituted with sterile water and dilutions were prepared from each sample using DMDM/10%FCS to yield final virus dilution (relative to the original culture supernatant) of 1/3.

## 3. <sup>3</sup>H Thymidine Incorporation Proliferation Assay.

To each final virus dilution human tonsillar B cells were added to  $10^6$ /ml and incubated for 90 minutes with gentle agitation to effect viral infection. The cells were then collected by centrifugation, resuspended at  $5 \times 10^5$ /ml and 0.2ml aliquots ( $10^5$  cells) plated/well of a 96-well flat tissue culture plate, three wells being plated for each condition. After 5 days incubation at 37°C 1μCi of <sup>3</sup>H Thymidine was added per well and after a 6 hour incubation the wells were harvested and incorporated <sup>3</sup>H Thymidine counted on a Beta counter.



## Results.

Sample	Cpm	%
EBV at 4°C	64528 ± 9702	(100)
EBV freeze dried	5641 ± 560	9
EBV + 1% trehalose	11860 ± 1466	18
EBV + 10% trehalose	37757 ± 5663	58.5
EBV + 20% trehalose	41793	65

Additionally, electron microscopy of negatively stained preparations showed that in the presence of 10% trehalose the ultrastructure of dried EBV was preserved, while in the absence of trehalose the ultrastructure is completely disrupted.

Example 2 - E. Coli bacteriophage (Lambda) GT10

## Background:

Bacteriophage lambda is capable of infecting E. Coli and entering either the lytic or the lysogenic pathway. In the lytic pathway the phage replicates in the host and eventually lyses the host, releasing all the phage. In the lysogenic pathway instead of replicating and lysing

the bacterial hosts the phage DNA enters the hosts genome and replicates with it. The gene responsible for controlling entry into lysogenic or lytic pathways is the phage repressor gene (C1). Phage  $\lambda$  carrying an insert, is seeded onto agar plates, the bacterial lawn contains clear areas (plaques) which are the sites of bacterial cell lysis due to phage replication and proliferation.

#### Experiment 1:

A single plaque was removed from an agar plate and stored in 1 ml of SM buffer (5.8gm NaCl, 2gm  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5ml of 1M Tris/HCl pH 7.5 and 5ml 2% gelatin made up to 1 litre) at 4°C.

A 1 in 1000 dilution was made from this stock in SM buffer. Aliquots of either 1 $\mu$ l or 10 $\mu$ l were taken and placed in polypropylene Eppendorf tubes with an equal volume of 20% trehalose in distilled water. Samples were dried at room temperature. The smaller volumes were dried in a laminar flow hood overnight and the larger volumes were dried in a dessicator attached to an evacuating air line. The samples were maintained in a dry state for 24-48 hours. For the plaque assay, 100 $\mu$ l of a suspension of E. Coli (approximately  $8 \times 10^7$  of strain NM 514) in 10mM  $\text{MgSO}_4$  was added to

each dried phage sample and incubated at 37°C for 30 minutes. After incubation, 10 mls of  $\lambda$  top agar (1% Bactotryptone, 0.5% Bacto-yeast extract, 0.5% NaCl, 0.25%  $\text{MgSO}_4$  and 1% (Bacto-agar) at 42°C was added to each sample plated out onto previously prepared L-Agar plates. These were left to cool, inverted and incubated at 37°C for approximately 16 hours, after which the number of plaques was counted.

#### Results:

Treatment of phage	Dilution of stock	Number of plaques/plate
Storage at 4°C in SM buffer	1 $\mu$ l of 1/1000 dilution	47
Dried in SM buffer	1 $\mu$ l of 1/1000 dilution	0
Dried in 10% trehalose	1 $\mu$ l of 1/1000 dilution	19 (40% plaquing efficiency of control)

#### Experiment 2

##### Long-Term Storage

The previous experiment gave details of how bacteriophage lambda carrying an insert can be dried in trehalose, with the preservation of function on rehydration. The bacteriophages in these experiments were rehydrated 24 to 48 hours after drying and were

shown to be capable of adsorbing and then penetrating an E.coli host bacterium and then replicating, maturing and finally lysing the host to release many new bacteriophages.

The following experiment tests the ability of trehalose to preserve bacteriophage lambda, so it can be rehydrated and still function, for longer periods and under adverse conditions. A 10% overall aliquot was taken from a stock of bacteriophage lambda, and placed in an Eppendorf tube along with an equal volume of 20% glucose or SM buffer or 20% trehalose or Luria Broth. The stock of bacteriophage lambda used was made by placing a single plaque from an agar plate culture in 1 ml of SM buffer. The samples were dried down in a desiccator attached to an evacuating air line, for at least 16 hours, and then the tops were screwed on the Eppendorfs. Until they were rehydrated the Eppendorfs containing the samples were kept under a variety of conditions. They were placed on the bench at normal room temperature which varied depending on the time of day and the seasons. They were kept in the dark or in direct sunlight. They were placed at 4°C. After 10 months they were rehydrated in a 100µl suspension of E.coli (approximately  $8 \times 10^7$ ) in 10mM  $MgSO_4$  and incubated following thorough mixing at 37°C for about 30 minutes. After incubation 4 mls of lambda top agar at 42°C was added to each sample and plated out onto

11

previously prepared L-agar plates. These were left to cool and then incubated at 37°C for approximately 16 hours. The number of plaques was counted.

Phage dried in following	Number of Plaques/Plate
10% Glucose	Nil
5M Buffer	Nil
Luria Broth	Nil
10% Trehalose	118

This gives a value of  $12 \times 10^6$  plaque forming units per ml of stock. This compares with  $47 \times 10^6$  pfu obtained in the assay carried out 10 months previously of the same stock and with  $19 \times 10^6$  pfu in samples rehydrated immediately after desiccation in trehalose buffer.

### Example 3

#### Poliovirus Type 3

50µl virus stock + 50µl 20% trehalose were dried at 37°C and stored at this temperature for either 24 hours or 7

days before rehydration and assay as compared with virus dried without trehalose and non-dried virus.

Figures shown are the drop in cytopathic titre in logs (base 10)

	24Hr	7D
With trehalose	3.4	3.7
Without trehalose	5.9	6.0
Non-dried	0.5	0.8

i.e trehalose shows a marked protective effect especially on storage at 37°C

Example 4

Influenza Virus strain X79 (A/Phil/2/82 x PR/8 H3N2) ER340

50µl virus + 50µl 20% trehalose were dried at room temperature and stored at this temperature for 48 hours then reconstituted with Dulbecco's MEM tissue culture medium, 10µl samples transferred to MDCK cells (FB24) and incubated for 48 hours. The haemagglutination titre was then checked.

	<u>Titre</u>
With trehalose	7.0
Without trehalose	4.5
Non-dried	7.0

i.e. trehalose protects completely at this temp. Other regimes of drying and storage with both polio and 'flu showed much less or no protection.

#### Example 5

##### Immunogenicity of Trehalose-dried Mink Enteritis Virus

Experiments done with UNITED VACCINES division of HARLAN SPRAGUE DAWLEY INC., Madison Wisconsin 53711 USA

United Vaccines BIOCOMP-DP is a combination vaccine of Mink Distemper-Enteritis virus Vaccines and Clostridium botulinum Type C with Pseudomonas aeruginosa as bacterin-toxoids. The only relevant data for this patent is the ability of the inactivated Mink Enteritis Virus (MEV) to immunise mink against challenge with live virulent enteritis virus. This component of the combined vaccine is sold as a liquid suspension of virions in which the other components of the vaccine, in lyophilised form, are resuspended immediately prior to injection.

The liquid MEV was lyophilised with and without 10% added trehalose and shipped to United Vaccines in Madison Wisc. who reconstituted the preparations with normal saline mixed in the dried components and assayed the vaccines for their ability to protect immunised mink against lethal challenge with live Mink Enteritis Virus

Live/Challenged

With trehalose	5/5
Without trehalose	1/5

i.e. the immunogenicity of this very desiccation-sensitive virus is preserved by trehalose.




CLAIMS:

1. A method of preserving live viruses comprising subjecting an aqueous system containing the virus to drying either in the frozen state or at ambient temperature, in the presence of trehalose.
2. A method according to claim 1 in which the aqueous system contains 1 to 20% by weight of trehalose.
3. A method according to claim 1 in which the aqueous system contains 5 to 20% by weight of trehalose.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00047

<b>I. CLASSIFICATION F SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC <sup>4</sup> : A 61 K 39/12; C 12 N 7/00; // A 61 K 35/76		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC <sup>4</sup>	C 12 N; A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	WO, A, 87/00196 (QUADRANT BIORESOURCES LTD) 15 January 1987 see page 5, lines 9-18; page 9, lines 3-9; claims 1,2,4 (cited in the application) --	1-3
A	EP, A, 0140489 (WAKO PURE CHEMICAL INDUSTRIES LTD) 8 May 1985, see page 1, lines 1-5; page 4, line 28 - page 5, line 4; page 7, line 18 - page 8, line 16; claims 1,4,5 (cited in the application) -----	1-3
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>14</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
10th May 1989	09.06.89	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 P.C.G. VAN DER PUTTEN	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 8900047

SA 26531

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 06/06/89  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8700196	15-01-87	AU-A- 6136386	30-01-87
		EP-A- 0229810	29-07-87
		GB-A- 2187191	03-09-87
		JP-T- 63500562	03-03-88
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EP-A- 0140489	08-05-85	JP-A- 60035263	23-02-85
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